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Tolerance and metabolic responses of Cyanidiophytina (Rhodophyta) towards exposition to Cl_4K_2Pd and $AuCl_4K$

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ABSTRACT

Polyextremophilic algae, such as unicellular red algae known as *Cyanidiophyceae*, have the intrinsic capacity to selectively mobilize and adsorb metals, since they are adapted to live in geothermal and volcanic sites characterized by elevated concentration of heavy and rare metals. In this work we evaluated the ability of 3 strains of the *genus Galdieria* (*G. maxima*, *G. sulphuraria*, *G. phlegrea*) along with one strain of *Cyanidium caldarium* to tolerate different concentrations of rare metal as Cl_4K_2Pd and $AuCl_4K$ by monitoring changes in algal growth in culture exposed to different concentration of each metal and investigating algae metabolic response and possible oxidative stress induced by these metals.

INTRODUCTION

In the last decade there is a remarkable and growing demand for Rare Earth Elements (REE) due to their large use in the superconductors, catalysts and electronic industry. On the other side, the issue of their discharge in the environment and the suitability of recycling REE from the electronic waste (e-waste) is of interest of all the population because of their hazard for environment and health, besides their economic value. These issues become evident to the government of the different States and electronic industries prone to develop new methods of removal from the environment, to recycle and re-input of REE in the productive cycle of a "closed loop economy"[1-4]. Recently, biological methods have been developed to ensure the recovery of small quantities of these mineral materials and wastewater systems, using mainly bacteria[5] or plants known for their ability to immobilize heavy metals in the cell wall and compartmentalization in vacuoles. Interestingly, polyextremophilic algae have the intrinsic properties that make them capable of selective removal and concentration of metals, thanks to their adaptation to live in geothermal and volcanic sites[6-8]. Geothermal fluids leach out of the hot volcanic rocks and are enriched by enormous amounts of minerals and metals, including lithium, sulfur, boric acid and precious metals such as gold, platinum, palladium and silver[9]. *Cyanidiophyceae*, unicellular red algae, survive in extreme conditions, very low pH (0.0-3.0) and high temperatures (37-55 °C), and colonize acid and hydrothermal sites, but also rocks and muddy soil around hot ponds[10]. They belong to 3 genera: *Cyanidioschyzon*, *Cyanidium* and *Galdieria*, which differ in size, cellular shape and growth conditions. *C. merolae* is the only species belonging to *Cyanidioschyzon* genus and differs from the other two groups being lacking of cell wall and dividing by binary fission[11]. Both *Cyanidium* and *Galdieria* are able to grow both on ammonia and nitrate, the former is an obligatory autotroph whereas the species belonging to *Galdieria* tolerate high concentrations of salts[12] and can grow autotrophically and heterotrophically, thus making *Galdieria* the best candidates for biotechnological application into the recovery of REE as it has been already proposed in previous studies from other groups[13,17]. These algae are one of the few eukaryotes capable of adapting to a very acidic environment. Because of the high temperature and acidic conditions, the environment that these algae live in is usually rich in metals; *Galdieria sulphuraria* is the most suitable alga of all of the Cyanidiophitina for biotechnology experiments and applications because it is the only one of these algae that can grow autotrophically as well as heterotrophically, using over 27 different kinds of sugar and polyols to produce a huge biomass and beneficial compounds[13]. In this report, we have evaluated the ability of *G. maxima*, *G. sulphuraria*, *G. phlegrea* and *C. caldarium* to tolerate different concentrations of REE (such as palladium-Cl_4K_2Pd and Gold-$AuCl_4K$) by analyzing Maximum Growth Rate (MGR) and the inverse of Generation Time (1/GT)[14]. We also investigated metabolic response and possible oxidative stress induced by the metals, by monitoring superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) activities[15, 16].

MATERIALS AND METHODS

Algal strains and culture conditions

The algal strains used in this study belong to the algal collection of the University of Naples (www.acuf.net), namely ACUF 3.4.5 (*G. maxima*), ACUF 7.6.21 (*G. phlegrea*), ACUF9.2.11 (*G. sulphuraria*) and ACUF 626 (*C. caldarium*). All strains were mantained in liquid culture in Allen medium[18], pH 1.5, at 37°C on a plexiglass shaking apparatus under a photon irradiance of 150 μmol photons m^{-2} s^{-1} with continuous light provided by cool-light fluorescent lamps (Philips TLD30w/55).

Experimental set up

3ml of each culture at exponential phase were transferred to a 24well-plate and the medium was supplemented with different metals concentrations (0,1gg/L-1g/L). Cell growth was assessed by recording the optical density (OD) at 750 nm (Bausch & Lomb Spectronic 20), at day 0 and day 4; MGR and 1/GT were calculated according to the formula $[(Log_{10}\ OD_{day4} - Log_{10}\ OD_{day0})/4]$ and $1/[Ln(2)/MGR]$, respectively. Three replicates were carried out for each test.

Enzyme activity

For measurement of enzyme activity algal cultures were transferred in falcon containing the metal at 1g/L in a final volume of 30 ml and pellet was harvested by centrifugation at 14000 rpm for 10min after 96h of exposure. Pellet was washed using KH_2PO_4 (0.1M pH7.8) followed by centrifugation at 12000 rpm for 4 min at 4°C, twice. Proteins were extracted homogenizing the sample with liquid nitrogen using mortar and pestel, resuspending the powder obtained in 3ml of Lysis Buffer (KH_2PO_4 0.5M pH7.8, DTT 2mM, EDTA 1mM, PMSF 1mM, PEG 1,25mM), samples were centrifuged at 14000rpm for 20 min at 4°C and the surnatant was used for measurement after Bradford quantification.

SOD measurement was determined adding 100µl of protein extract to the buffer containing KH_2PO_4 50mM pH7.8, Na-EDTA 0.1mM pH7.0, Metionin 13mM, Nitro Blue Tetrazolium (NBT) 75µM, Riboflavin 2µM), after 15min exposure to the light samples were read at 560nm using a Spectrophotometer. Enzymatic activity was expressed as units/g of dry weight. CAT measurement was performed adding 200µl of protein extract to the buffer containing KH_2PO_4 50mM pH7.8, H_2O_2 20mM and H_2O up to 1ml. Absorbance was recorded at 240nm for 100 sec. Enzymatic activity was expressed as nmol H_2O_2/g of fresh weight. APX measurement was obtained adding 100µl of protein extract to the buffer containing KH_2PO_4 100mM pH7, Na-EDTA 0,66mM, Ascorbic Acid 0.33mM, H2O2 0.35mM. Absorbance at 290nm for 100sec was recorded. Enzymatic activity was expressed as µmol ascorbate/g of fresh weight.

Each condition for each experimental approach was tested 3 times independently.

RESULTS AND DISCUSSION

Cyanidiophyceae are polyextremophilic micro-algae with an intrinsic ability to uptake metals, involving both active and passive mechanisms. Heavy, rare or precious metals can influence algae physiology in various ways, likely inhibiting different physiological processes. In order to evaluate the suitability of *Cyanidiophyceae* for biotechnological application to effectively recover REE, we tested their tolerance to Cl_4K_2Pd and $AuCl_4K$ monitoring the growth and metabolic response of 4 different strains, exposed to each of these metals ranging from 0.1g/L up to 10g / L. The growth was evaluated after 4 days since the single metal exposure and the results are expressed in the form of maximum growth rate (MGR) and the inverse of the Generation Time (1 /GT). *G. maxima* was more sensitive to $AuCl_4K$ showing a continuous decline in growth rate (Fig. 1A), while the growth of *G. maxima* declined at Cl_4K_2Pd 0,1g/L then sharply increased at higher concentrations (Fig.1A). both gold and palladium induced a significant decrease in growth rate of *G. phlegrea* from 0.1g/L to 10g/L (Fig.1B). As shown in Fig.1C, the MGR and 1/GT in *G. sulphuraria* dropped at 0,1g /L in both metals; the algal growth was steady until 1g/L $AuCl_4K$, then sharply dropped at higher concentration; viceversa, the growth rate significantly and progressively increased at palladium concentrations from 1 to 10 g/L. *C. caldarium* showed a high tolerance to Cl_4K_2Pd whereas $AuCl_4K$ inhibited algal growth with the increase of metal concentration (Fig.1D).

ROS scavenging activities of SOD, CAT and APX were assessed in all algae under Cl_4K_2Pd and $AuCl_4K$ at a concentration of 1g/L, after 24 hours. The antioxidant activity can be considered a measure of the effectiveness of the cell to respond to the impact of the metal, increasing its tolerance as protective mechanisms necessary to remove ROS before they can damage sensitive parts of the cellular machinery[16]. SOD catalyses the dismutation of O_2 (singlet oxygen) to O_2 and H_2O_2, representing the first line of cell defense against ROS production; CAT catalyses the production of H_2O from the degradation of H_2O_2 and ROOH; APX reduces H_2O_2 to H_2O using the ascorbate as an electron donor. The strain/metal specific metabolic responses were quite different as shown in Fig.2. Indeed, a slight increase in enzymatic activity was recorded in *G. maxima* APX and SOD as a response to Cl_4K_2Pd; CAT activity significantly decreased both under Cl_4K_2Pd and $AuCl_4K$ (Fig.2A). SOD, CAT and APX activities decreased in presence of Cl_4K_2Pd while increased in presence of $AuCl_4K$ in *G. phlegrea* (Fig.2B); a decrease in all enzymatic activities were recorded in *G. sulphuraria* (Fig.2C); a significant increase in all the enzymatic activities were recorded in *C. caldarium* only under Cl_4K_2Pd (Fig.2D).

A significant increase of the enzymatic activity compared to the control suggests a high scavenging activity of the singlet oxygen in peroxide of hydrogen, which can be expressed as an obvious tolerance of these algae to the metal under examination. An increase in the activity of both antioxidant enzymes is necessary to reduce the concentrations of both singlet oxygen and hydrogen peroxide, minimizing the risks. In general, the modulation of antioxidant enzymes is an important adaptive response to counteract adverse conditions; in fact, the maintenance of a high antioxidant capacity in the cells can be correlated with an increased tolerance against different types of environmental stress[15, 16, 19].

CONCLUSIONS

Our results showed a higher tolerance to Cl_4K_2Pd *vs* $AuCl_4K$ in the cyanidiophyceaen strains used in the present work. The growth and the metabolism of *G. phlegrea* was more affected by the presence of both metals. The contribute to the oxidative equilibrium of these polyextremophilic microalgae and the induction of antioxidant enzymes could result from the adaptation of the cell to the development of intracellular ROS; however there is not a clear correlation between any of the enzymatic activity and the better performing growth of the other 3 strains tested. The study from Ju and coworkers[17] showed the ability of *G. sulphuraria* in efficiently recovering both Cl_4K_2Pd and $AuCl_4K$, without assessing the influence of both metals on growth and physiology. We considered that tolerance is an essential parameter to take into account for biotechnological application, such as REE recovering. Our observations strongly suggest that other strains than *G. sulphuraria* can be used to recover REE, due to their high tolerance to precious and heavy metals, moreover further studies will be necessary to clarify the biological mechanisms underlying the tolerance capacity of *Cyanidiophyceae* and their strategies to respond to the metal toxicity in order to considerer by biotechnological future application.

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Figure 1. Evaluation of metal tolerance monitoring MGR and 1/GT at day 4

Maximum growth rate (MGR, left) and inverse of Generation Time (1/GT, right) measured at day 4 in G.*maxima* (A), G.*phlegrea* (B), G.*sulphuraria* (C) and C.*caldarium* (D) grown at different concentrations of palladium and gold. MGR and 1/GT in presence of different concentration of palladium (Cl_4K_2Pd, orange line/bar) and gold ($AuCl_4K$, blue line/bar). Error bars represent standard deviation of three replicate cultures; (*) = p-value ≤ 0,001calculated by T-test.

Figure 2. Evaluation of enzymatic activities after metals exposition

Enzymatic activity measured in G.*maxima* (A), G. *phlegrea* (B), G. *sulphuraria* (C) and C. *caldarium* (D) treated by 1g/L of palladium (Cl_4K_2Pd, orange bar) and gold ($AuCl_4K$, blue bar) after 96h, Relative units represent: units/g of dry weight (SOD); nmol H_2O_2/g of fresh weight (CAT); μmol ascorbate/g of fresh weight (APX) Mean (± SD) was calculated from three replicates. (*) = p-value ≤ 0,05 calculated by T-test.

Fig.1

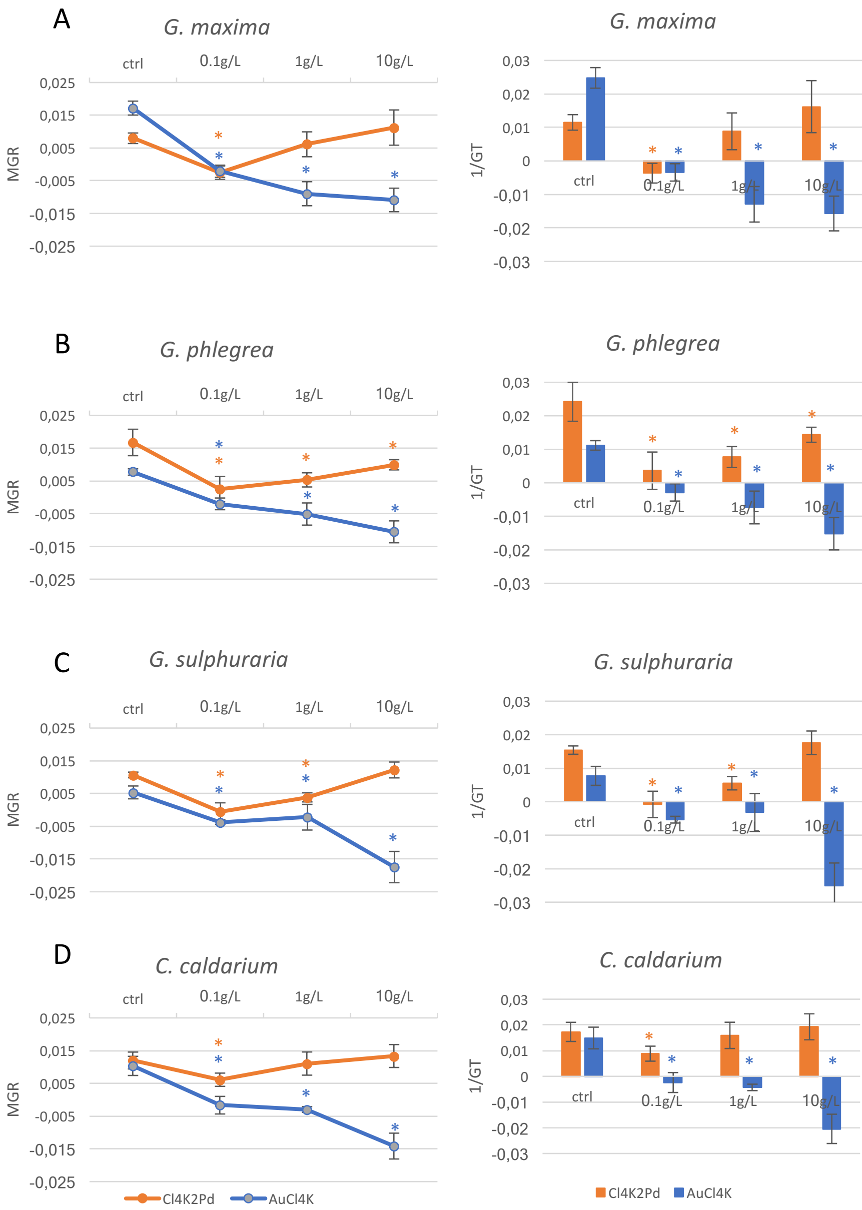
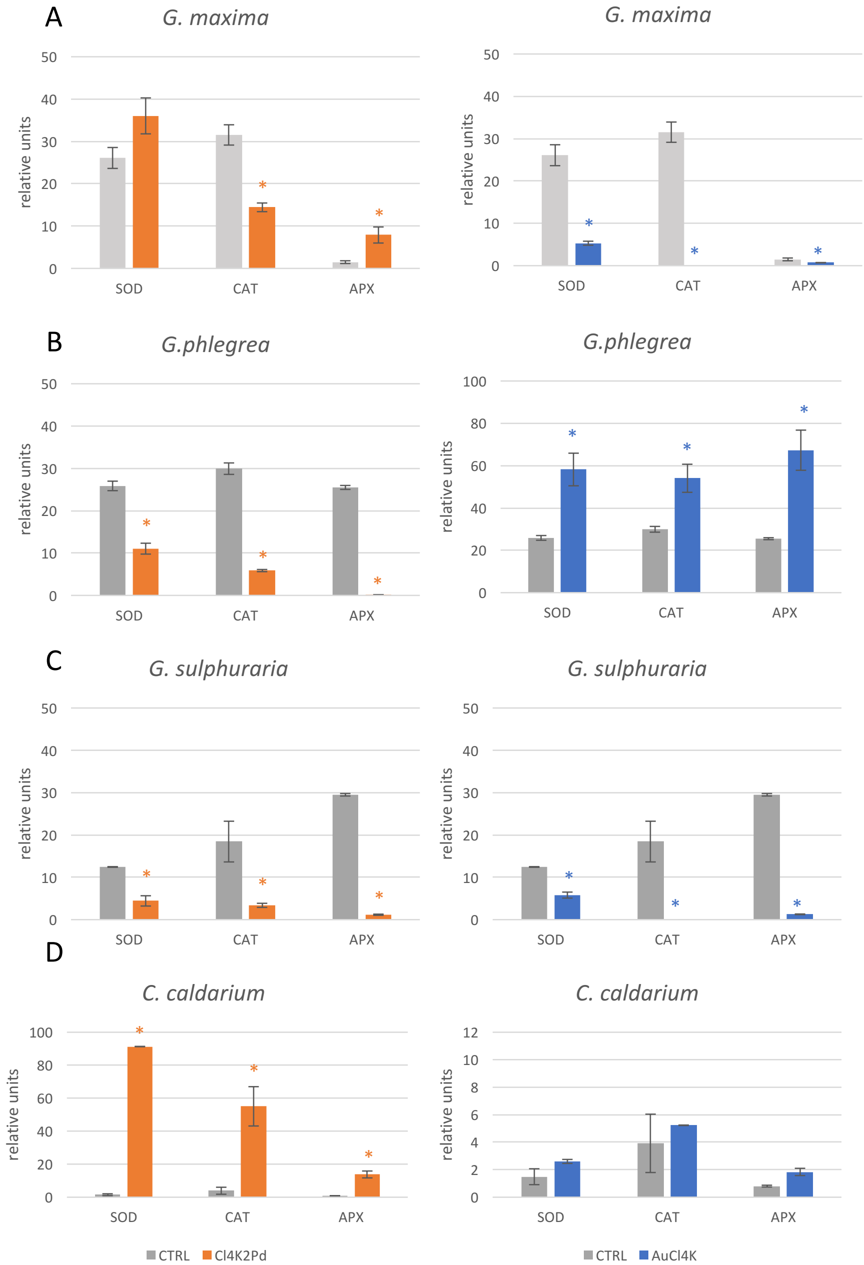

A
G. maxima
B
G. phlegrea
C
G. sulphuraria
D
C. caldarium
ctrl
0.1g/L
1g/L
10g/L
MGR
0,025
0,015
0,005
-0,005
-0,015
-0,025
1/GT
0,03
0,02
0,01
0
-0,01
-0,02
-0,03
*
Cl4K2Pd
AuCl4K

Fig.2


A
G. maxima
relative units
50
40
30
20
10
0
SOD
CAT
APX
G. maxima
B
G.phlegrea
100
80
60
40
20
C
G. sulphuraria
D
C. caldarium
12
10
8
6
4
2
CTRL
Cl4K2Pd
AuCl4K